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Alcohol Stimulates Estrogen Receptor Signaling in Human Breast Cancer Cell Lines[1]

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Abstract

Epidemiological studies suggest that moderate alcohol consumption increases the risk of breast cancer, and that alcohol combined with estrogen replacement therapy may synergistically enhance the risk. However, the mechanism(s) of alcohol-induced mammary cancer is unknown. In human breast cancer cell lines, we found that ethanol (EtOH) caused a dose-dependent increase of up to 10- to 15-fold in the transcriptional activity of the liganded estrogen receptor (ER-α), but did not activate the nonliganded receptor. Significant stimulation of ER-α activity was observed at EtOH concentrations comparable with or less than blood alcohol levels associated with intoxication and at doses below the threshold for *in vitro* cytotoxicity. These findings may be explained, in part, by an EtOH-induced down-regulation of the expression of BRCA1, a potent inhibitor of ER-α activity, and, in part, by a modest increase in the ER-α levels. Our findings suggest that inactivation of *BRCA1* and increased estrogen-responsiveness might contribute to alcohol-induced breast cancer.

Introduction

Epidemiological studies have documented a relationship between moderate alcohol consumption and breast cancer rates in women (1–3). In a pooled analysis of six prospective cohort studies that examined dietary factors in breast cancer, increasing alcohol intake correlated significantly with the breast cancer risk (1). Previous studies had suggested that the combination of alcohol consumption plus postmenopausal estrogen replacement therapy synergistically enhances the risk of cancer (reviewed in Refs. 4 and 5). Several studies report increased levels of circulating estrogen associated with alcohol use, but other studies have failed to demonstrate an increase in circulating or urinary estrogen in response to alcohol use (reviewed in Ref. 6).

Aside from ionizing radiation, alcohol consumption is probably the best-defined environmental risk factor for breast cancer, but the mechanism(s) of alcohol-induced carcinogenesis is not understood (7, 8). In combination with other events such as oncogenic mutations and inactivation of tumor suppressors, prolonged estrogenic stimulation of the mammary epithelia is thought to contribute to the development of breast cancer. In this report, we show that alcohol can down-regulate the tumor suppressor BRCA1 (9) and stimulate ER-α[3] activity, both of which might contribute to alcohol-induced breast cancer.

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[3] ER, estrogen receptor; wt, wild type; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ERE, estrogen-responsive enhancer; RT-PCR, reverse transcription-PCR.

Materials and Methods

Expression Vectors and Reporters. The wt BRCA1 expression plasmid was created by cloning the BRCA1 cDNA into the pcDNA3 mammalian expression vector (Invitrogen) using artificially engineered 5′ *Hin*dIII and 3′ *Not*I sites. The wtBRCA1 plasmid was provided by Michael Erdos (National Human Gene Research Institute, NIH, Bethesda, MD). The expression vector pCMV-ER-α was used to express ER-α. The estrogen-responsive reporter plasmid ERE-TK-Luc is composed of the vitellogenin A2 ERE controlling a minimal thymidine kinase promoter (TK81) and luciferase, in plasmid pGL2 (10).

The E2F reporter (E2F-TK-Luc) is composed of the E2F site from adenovirus E2a linked to the minimal TK promoter (TK81) and luciferase, and the Sp1 reporter (Sp1-TK-Luc) is composed of the Sp1 site from the cyclin D1 promoter (−127 to −99), TK81, and luciferase. Expression plasmids for E2F1 (pCMV-E2F1) and Sp1 (pCMV-Sp1) and the E2F- and Sp1-responsive reporter plasmids were provided by Dr. Richard Pestell (Departments of Medicine and Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY).

Cell Lines. Hormone-responsive human breast cancer cell lines MCF-7 and T47D were obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 5% FCS, L-glutamine (5 mM), nonessential amino acids (5 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml; Ref. 11).

Estrogen Receptor (ER-α) Transcriptional Assays. ER-α transcriptional activity was determined by measuring the estrogen-stimulated, ER-α mediated activation of the estrogen-responsive reporter plasmid ERE-TK-Luc. Assays were performed essentially as described earlier (11). Briefly, asynchronously proliferating cells at about 50–70% of confluency in 24-well dishes were washed several times and incubated overnight with 0.25 μg of each vector in serum-free DMEM containing Lipofectin (Life Technologies). Cells were then washed at least three times, incubated in serum-free, phenolphthalein-free DMEM (0.2 ml/well) without or with 17β-estradiol (E2, 1 μM) and/or ethanol for 24 h, and harvested for luciferase assays. Luciferase values are means ± SE of four replicate wells and are representative of several independent experiments. In some experiments, plasmid pRSV-β-gal was cotransfected as a control for transfection efficiency. These experiments revealed no effect of ethanol or wtBRCA1 on β-galactosidase activity.

Assays of Cytotoxicity. MTT Assays. MTT assays of cell viability were performed as described previously (12). This assay is based on the ability of viable cells to convert MTT, a soluble tetrazolium salt, into an insoluble formazan precipitate, which is quantitated by spectrophotometry after solubilization in DMSO (13). Briefly, subconfluent proliferating cells in 96-well dishes were treated with different doses of ethanol for 24 h in serum-free DMEM, after which the cells were solubilized and absorbance readings were taken using a multiwell spectrophometer. The amount of MTT dye reduction was calculated based on the difference between absorbance at 570 nm and at 630 nm. Cell viability was expressed as the amount of dye reduction relative to that of untreated controls.

Apoptosis Assays. Subconfluent exponentially proliferating cells in 100-mm plastic Petri dishes were incubated with different doses of ethanol in serum-free DMEM for 24 h, and then the cells were counted using a hemacytometer. Samples were normalized by cell number (500,000–750,000 cells), and the low molecular weight apoptotic DNA was extracted as described previously (12, 14). The DNA was electrophoresed through 1.2% agarose gels

containing 0.1 mg/ml of ethidium bromide, and the gels were photographed under UV illumination.

Western Blotting. Preparation of whole cell lysates and Western blotting was performed as described previously (12). The primary antibodies and their sources were as follows: (*a*) BRCA1 (C-20, rabbit polyclonal, Santa Cruz Biotechnology, 1:200 dilution); (*b*) ER-α (H-184, rabbit polyclonal, Santa Cruz, 1:1000); (*c*) Bax (P-19, Santa Cruz); (*d*) Bcl-2 (N-19, Santa Cruz); and (*e*) α-actin (I-19, goat polyclonal, Santa Cruz, 1:500). Proteins were visualized using the enhanced chemiluminescence detection system (Amersham), with colored markers (Bio-Rad) as size standards.

Semiquantitative RT-PCR Analysis. The BRCA1 mRNA expression was evaluated by semiquantitative RT-PCR, as described previously by us (12). Briefly, total cell RNA was extracted from the cell monolayers using TriPure reagent (Boehringer Mannheim), treated with DNase, and purified by phenol-chloroform extraction. Aliquots of RNA (5 μg) were reverse-transcribed using Superscript II reverse transcriptase (Life Technologies, 10,000 units/ml). Aliquots of cDNA corresponding to 0.5 μg of original RNA were used for PCR amplification. The cycle number ($n = 27$) was adjusted so that all reactions fell within the linear range of amplification. The PCR primers and predicted products were as follows: BRCA1, 5′TTGCGGGAGGAAAATGGGTAGTTA′3 (forward), 3′TGTGCCAAGGGTGAATGATGAAG′5 (backward), 285 bp (position in DNA 5239-5524); and β-actin, 5′TTGTTACCAACTGGGACGATA3′ (forward), 3′GATCTTGATCTTGGTGCT5′ (backward), 764 bp (position in DNA 265-1028).

Results

Alcohol Stimulates the Transcriptional Activity of ER-α in Cultured Human Breast Cancer Cells. To determine whether ethanol could alter the mammary cell sensitivity to estrogen, we performed studies to investigate whether ethanol affects the transcriptional activity of ER-α. Initially we tested MCF-7 cells, a commonly studied human breast cancer cell line that is estrogen- and progesterone-receptor-positive and wt for the *p53*, *Rb*, and *BRCA1* tumor suppressor genes. Cells were transfected with ER-α, to ensure high-level ER expression (see below) and an estrogen-responsive reporter plasmid (ERE-TK-Luc) and assayed for estradiol (E2)-stimulated reporter activity. E2 alone induced an ~100-fold stimulation of reporter activity in MCF-7 cells (positive control).

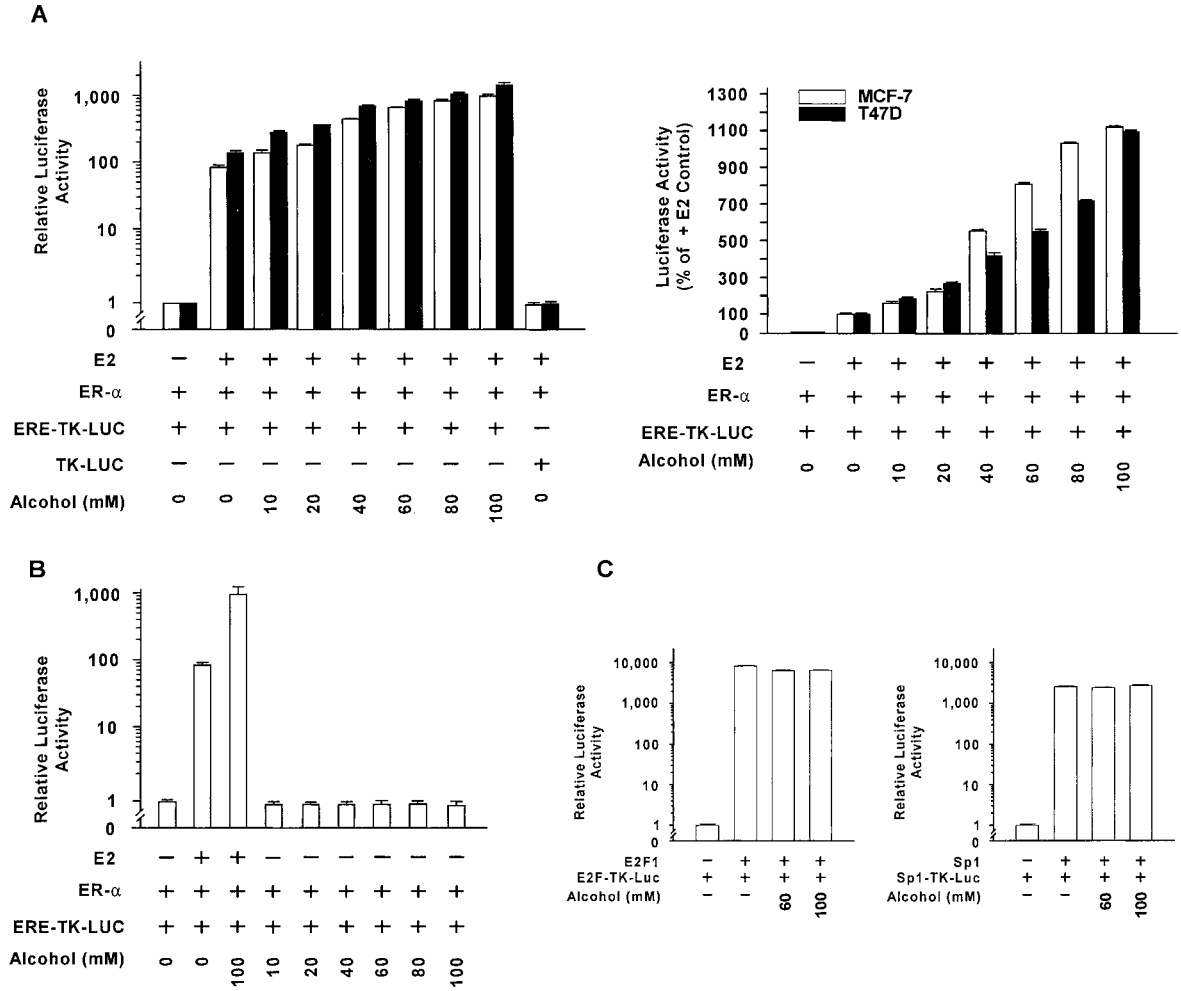

Fig. 1. Alcohol stimulates the ER-α transcriptional activity in human breast cancer cells. *A*, alcohol causes dose-dependent stimulation of the activity of the liganded ER-α. Estrogen-responsive breast cancer cell lines MCF-7 and T47D were assayed for stimulation of ERE-TK-Luc reporter activity by 17β-estradiol (E2), as described in "Materials and Methods." An ER-α expression plasmid pCMV-ER-α ("ER-α") was provided to ensure high level expression of ER-α under all assay conditions. After an overnight transfection of ER-α plus ERE-TK-Luc, cells were washed and incubated with E2 plus different doses of alcohol (ethanol) for 24 h before harvesting for luciferase assays. Results are shown on logarithmic (*left*) and linear (*right*) scales. Luciferase activity is expressed relative to the −E2 negative control or as a percentage of the (+E2; 0 ethanol) positive control, normalized to β-galactosidase activity. Values are means ± SE of quadruplicate determinations. For ethanol doses ≥20 mM, luciferase activities were significantly greater than the control (+E2; 0 alcohol): $P < 0.001$ (two-tailed *t* test) for each cell line. *B*, alcohol does not stimulate the activity of the nonliganded ER-α. In the absence of E2, reporter activity was not increased above baseline at any ethanol dose in MCF-7 cells ($P > 0.1$). Luciferase values are expressed relative to the (−E2; 0 ethanol) negative control and are normalized to β-galactosidase activity. *C*, alcohol does not stimulate the transcriptional activity of E2F1 or Sp1 in MCF-7 cells. The effect of ethanol on E2F1 and Sp1 activity was examined using expression vectors pCMV-E2F1 ("E2F1") or pCMV-Sp1 ("Sp1") and E2F- or Sp1- responsive luciferase reporters. The basal activity of each reporter was very low, but was stimulated by E2F1 or Sp1 vector ($P < 0.001$; two-tailed *t* test). Neither E2F1 nor Sp1 stimulated reporter activity was altered by ethanol ($P > 0.1$).

In cells incubated with E2 plus ethanol for 24 h, there was a dose-dependent increase in E2-stimulated reporter activity, as compared with the positive control (*i.e.*, cells incubated with E2 but no ethanol). This effect seems to be more dramatic when plotted on a linear (Fig. 1*A*, *right*) than on a logarithmic scale (Fig. 1*A*, *left*). Generally similar results were obtained using MCF-7 and T47D cells. In MCF-7, the maximum stimulation of ER-α activity was ~10-fold, relative to the positive control (+E2, 0 ethanol) and was observed at 80–100 mM ethanol. In T47D, the maximum ethanol-induced stimulation was 13-fold and occurred at 200 mM ethanol. At doses higher than 100 mM in MCF-7 and 200 mM in T47D there was a reduction of ER-α activity consistent with cytotoxicity (see below).

As a control, the same reporter plasmid missing the estrogen-responsive element (TK-Luc), showed very low basal luciferase activity and no estrogen stimulation of activity (Fig. 1*A*). In contrast to E2-stimulated cells, cells incubated with ethanol for 24 h in the absence of E2 showed no ethanol-stimulated ERE-TK-Luc reporter activity at any dose of ethanol (Fig. 1*B*). Thus, the ability of ethanol to stimulate ER-α activity in human breast cancer cells is specific to liganded ER-α.

Although most experiments were performed with transfected ER-α to ensure high level ER-α expression, we tested the effects of two doses of ethanol (60 mM and 100 mM) in MCF-7 and T47D cells in the absence of exogenous ER-α. When normalized to the +E2, 0 ethanol control (=100%), the luciferase activities (normalized to cotransfected β-gal activity) at 60 mM and 100 mM of ethanol were as follows: MCF-7, 437% and 981%, respectively; and T47D, 389% and 528%, respectively (SE, <5%). These values were significantly higher than the controls ($P < 0.001$, two-tailed *t* tests), indicating that it is not necessary to supply exogenous ER-α to demonstrate the stimulation of ER-α transcriptional activity by ethanol.

Doses of ethanol that markedly enhanced liganded ER-α transcriptional activity (60–100 mM) did not stimulate the activity of two cell cycle-regulated transcription factors, E2F1 and Sp1, as demonstrated by assays using E2F- and Sp1-responsive reporters (Fig. 1*C*). These findings suggest that the ability of ethanol to activate ER-α is not because of nonspecific transcriptional activation.

It is not likely that the ethanol-induced alterations of ER-α activity were attributable to toxicity, because ER-α activity was increased rather than decreased, within the range of ethanol concentrations comparable with achievable blood alcohol levels. However, to determine the dose-effect relationship for alcohol toxicity, MCF-7 cells were treated with ethanol for 24 h and assayed using: (*a*) the MTT assay, a spectrohotometric assay of cell viability based on the ability of intact mitochondria to reduce a tetrazolium dye to formazan; and (*b*) agarose gel electrophoresis to assess the presence of low molecular weight interoligosomal DNA fragments ("DNA ladders") characteristic of apoptosis.

At <100 mM of ethanol, cell viability determined by the MTT assay was >95%; whereas concentrations ≥100 mM of ethanol caused a dose-dependent reduction of cell viability from 90% (100 mM) down to 65% (500 mM). Agarose gel electrophoresis revealed apoptotic DNA ladders at ethanol concentrations ≥100 mM, with little or no evidence of laddering at lower doses of ethanol (data not shown). These findings suggest that toxicity is not a major contributory factor to ethanol-induced cellular alterations at concentrations <100 mM of ethanol.

Alcohol Partially Reverses the BRCA1-mediated Inhibition of ER-α Transcriptional Activity. We recently reported that BRCA1 inhibits ER-α signaling in various human breast cancer cell lines, including MCF-7 and T47D cells (11). To determine whether ethanol could overcome the BRCA1-mediated repression of ER-α activity, ER-α/ERE-TK-Luc transcriptional assays were performed in MCF-7 cells cotransfected without or with a wtBRCA1 expression vector. In the experiment shown in Fig. 2*A*, E2 induced a 60-fold increase in luciferase activity (relative to the −E2 control), and wtBRCA1 caused inhibition of E2-stimulated ER-α activity nearly down to the −E2 control levels. E2-stimulated ER-α activity (relative luciferase activity of 40) was observed in wtBRCA1-transfected cells exposed to ethanol (100 mM), suggesting that ethanol opposes the BRCA1-mediated repression.

Whereas ethanol stimulated ER-α activity in wtBRCA1-transfected cells, the relative luciferase activity was much lower in wtBRCA1-transfected, ethanol-treated cells (40) than in nontransfected, ethanol-

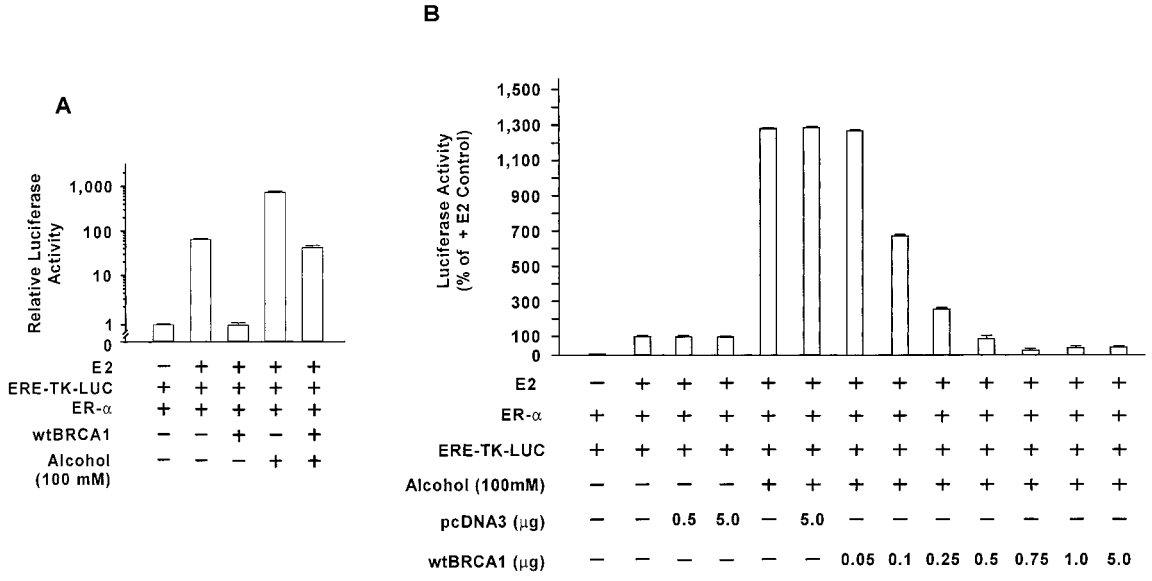

Fig. 2. Alcohol partially reverses the BRCA1-mediated repression of ER-α transcriptional activity in MCF-7 cells. *A*, cotransfection of a wtBRCA1 expression plasmid (wtBRCA1) virtually abrogated the E2-induced activation of reporter ERE-TK-Luc. However, in the presence of 100 mM of ethanol, the ability of wtBRCA1 to repress ER-α activity was significantly attenuated; the relative luciferase activity (+E2, +wtBRCA1) was significantly greater in the presence than in the absence of ethanol ($P < 0.001$; two-tailed *t* test). *B*, dose-dependent inhibition by wtBRCA1 of the ability of ethanol (100 mM) to stimulate ER-α activity. For each wtBRCA1 plasmid dose ≥0.1 μg, the +E2 luciferase activity was significantly reduced, as compared with that in the absence of wtBRCA1 ($P < 0.001$). Note that the empty BRCA1 vector (pcDNA3) had no effect on ER-α activity in the absence or presence of ethanol ($P > 0.1$).

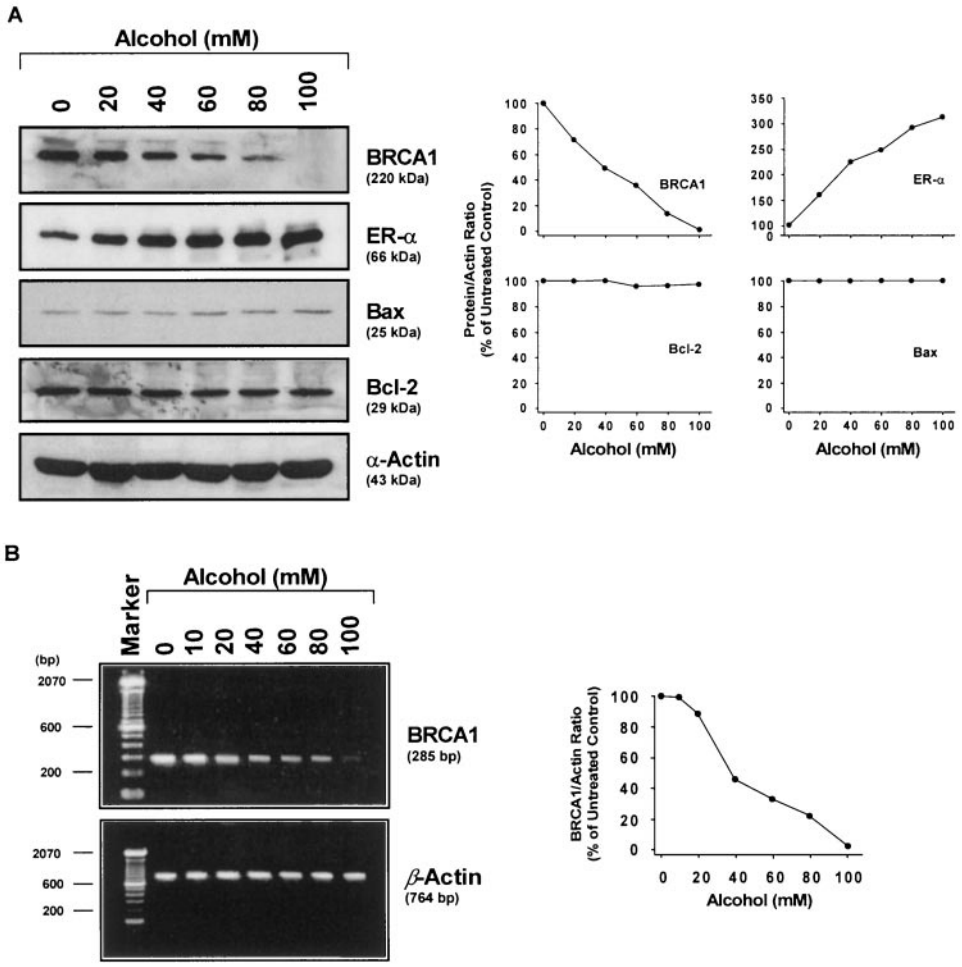

Fig. 3. Alcohol induces dose-dependent alterations in BRCA1 and ER-α levels in MCF-7 cells. *A*, effect of alcohol on protein levels. Subconfluent proliferating cells were incubated in the presence of different concentrations of ethanol in serum-free culture medium (DMEM) for 24 h, to match conditions used for the transcriptional assays shown in Fig. 1. Cells were then harvested, and equal aliquots of total cell protein (50 μg/lane) were Western blotted. Protein bands were quantitated by densitometry and expressed relative to the 43-kDa α-actin control band. *B*, effect of alcohol on BRCA1 mRNA levels determined by semiquantitative RT-PCR analysis. Cells were treated with ethanol as described above and harvested for semiquantitative RT-PCR assays. The 285-kDa amplified BRCA1 product was quantitated by densitometry and expressed relative to the control gene, *β-actin*. These assays revealed a dose-dependent decrease in BRCA1 mRNA expression, relative to β-actin.

treated cells ($\cong$700). Stated in other terms, over-expression of wtBRCA1 blocked the ability of ethanol to stimulate ER-α activity. As illustrated in the plasmid dose-response study in Fig. 2*B*, wtBRCA1 caused dose-dependent inhibition of ER-α activity in the presence of 100 mM of ethanol, ultimately down to or below the positive control (+E2, 0 ethanol) level in MCF-7 cells.

Alcohol Down-Regulates BRCA1 and Up-Regulates ER-α Expression in MCF-7 Cells. Subconfluent proliferating MCF-7 cells were incubated with different doses of ethanol for 24 h and then harvested for Western blotting, to determine the effect on the levels of BRCA1, ER-α, and other proteins. Protein bands were quantitated by densitometry and expressed relative to α-actin, as the control. This experiment revealed a dose-dependent decrease in BRCA1 to $<$5% of control and a dose-dependent increase in ER-α protein by about 3-fold at 100 mM of ethanol (Fig. 3*A*). Alterations in BRCA1 and ER-α protein levels were observed at ethanol concentrations as low as 20–40 mM. These concentrations are within the range of blood alcohol levels achieved by acute alcohol consumption: a blood alcohol level of 0.2% ("legally drunk") corresponds to 43 mM ethanol. Furthermore, these ethanol concentrations are below the threshold required to cause cytotoxicity of MCF-7 cells (100 mM; see below). In contrast to BRCA1 and ER-α, levels of the proapoptotic protein Bax, the antiapoptotic protein Bcl-2, and α-actin were unchanged.

We described a very sensitive method to measure BRCA1 mRNA by semiquantitative RT-PCR analysis (12). Semiquantitative RT-PCR assays of MCF-7 cells exposed to ethanol for 24 h revealed a dose-dependent decrease in BRCA1 mRNA at ethanol concentrations $\geq$20 mM, with no change in mRNA levels of the control gene, *β-actin* (Fig. 3*B*). Thus, the ethanol-induced decrease in BRCA1 was observed at both the mRNA and the protein levels.

Discussion

Alcohol is an etiological agent for several different tumor types, including upper aerodigestive cancers (mouth, oropharynx, hypopharynx, and esophagus) and breast cancer (15, 16). Alcohol is metabolized by the microsomal ethanol-oxidizing system, the activity of which is enhanced by chronic alcohol use (reviewed in 17). Because the microsomal ethanol-oxidizing system, including cytochrome P450 (CYP), has a major role in oxidative metabolism of environmental agents such as components of cigarette smoke, its enhanced activity may contribute to metabolism of aryl hydrocarbons present in smoke to active carcinogens. However, tobacco use is not known to be a major risk factor for breast cancer (17). Alcohol is coveted by alcohol dehydrogenase to acetaldehyde, which can induce DNA damage (18). This mechanism does not explain the specific association of alcohol with breast cancer, inasmuch as alcohol-induced DNA damage should occur in all cell types. The goal of these studies was to establish a mechanism to explain the association between alcohol use and breast cancer by focusing on breast cancer-specific cellular and molecular alterations.

The role of alcohol as a mammary carcinogen is suggested by epidemiological studies, but there are, as yet, no compelling data to indicate a mechanism for alcohol-induced breast cancer (reviewed in Ref. 8). Furthermore, the mechanism of alcohol-induced breast cancer may be different from that of head and neck cancers, in which the

primary carcinogens may originate as procarcinogens in tobacco smoke. Our studies suggest a potential mechanistic linkage of alcohol and breast cancer by documenting effects of ethanol on two molecular pathways directly related to breast cancer: estrogen response and BRCA1 function.

Thus, ethanol stimulated the transcriptional activity of the liganded estrogen receptor (ER-α) in human breast cancer cell lines, although it did not cause *de novo* activation of ER-α in the absence of the ligand, estrogen. The stimulation of ER-α activity by ethanol was observed at concentrations of ethanol comparable with those achieved during intoxication. Thus, 40 mM ethanol, which gave a significant stimulation of ER-α in MCF-7 and T47D cell cultures, corresponds to a blood alcohol level of about 0.2. Stimulation of ER-α activity was observed at ethanol doses lower than the threshold for cytoxicity (about 100 mM in MCF-7 cells); and ethanol did not induce the activation of two other cellular transcription factors: E2F1 and Sp1. Taken together, these findings suggest that ethanol may cause physiologically relevant stimulation of ER-α activity, and that the stimulation is not attributable to nonspecific actions.

Mutations of the breast cancer susceptibility gene *BRCA1* (17q21) confer an increased risk for breast and ovarian cancers (9, 19). *BRCA1* encodes an 1863 amino acid, 220 kDa nuclear phospho-protein with an N-terminal RING finger domain that interacts with cell cycle proteins and an acidic COOH-terminal transcriptional activation domain (9, 20, 21). BRCA1 plays roles in cell cycle regulation, apoptosis, and DNA repair and recombination pathways that may be related to its tumor suppressor function (reviewed in Ref. 22). The finding that ethanol down-regulates the mRNA and protein levels of BRCA1 in human breast cancer cells suggests a second possible mechanism linking ethanol to breast cancer: *i.e.*, down-regulation of the *BRCA1* tumor suppressor gene. Interestingly, a significant fraction of sporadic human breast cancers contain decreased levels of immunoreactive BRCA1 (23), suggesting that decreased BRCA1 expression is a mechanism through which sporadic breast cancers may escape the control of this tumor suppressor in the absence of an inactivating mutation.

The ability of ethanol to up-regulate ER-α expression and to down-regulate BRCA1 expression may each contribute to the stimulation of ER-α transcriptional activity. It is unlikely that the increase in ER-α activity could be explained solely by an increased level of ER-α protein, for several reasons: (*a*) the increase in ER-α activity in the transcriptional assays ($\geq$10-fold at 100 mM ethanol) was greater than the increase in ER-α protein ($\cong$3-fold at 100 mM ethanol); and (*b*) although we did not measure ER-α protein levels in the transcriptional assays, an ER-α expression vector was used to equalize ER-α levels to the extent possible in cells treated without or with ethanol.

Inasmuch as BRCA1 is a potent repressor of ER-α transcriptional activity (11), the ethanol-mediated down-regulation of BRCA1 expression could contribute to increased intrinsic activity of ER-α independently of any changes in ER-α levels. In this study, ethanol partially overcame the inhibition of ER-α activity caused by overexpression of a *wtBRCA1* gene; and conversely, expression of the *wtBRCA1* gene caused dose-dependent loss of ER-α activity in the presence of ethanol.

In evaluating these findings, it should be noted that we do not know what levels of ethanol are achieved in human mammary tissue after alcohol ingestion; nor do we know if the effects of acute *versus* chronic ethanol exposure on ER-α function are different. However, previous studies indicate that relatively high levels of ethanol (44–88% of serum levels) accumulate in the milk of lactating rats (24), and it is well established that sufficiently high levels of toxins, including alcohol, cocaine, etc., can be found in human breast milk to cause toxicity or even death to the baby (25, 26). These considerations suggest that ethanol may accumulate in considerable levels in the mammary tissue.

The molecular mechanisms underlying the ethanol-induced alterations of ER-α activity and BRCA1 expression and their significance need to be elucidated further. However, this study suggests that decreased expression of BRCA1 and increased estrogen-responsiveness might contribute to alcohol-induced breast cancer, and it provides directions for additional research.

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